Mater. Res. Soc. Symp. Proc. Vol. 845 © 2005 Materials Research Society

AA1.8

Competitive Guidance Cues Affect Fibroblast Cell Alignment: Electric Fields vs. Contact Guidance

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ABSTRACT

When bovine ligament fibroblast cells were cultured on parallel micro-grooved surfaces, they aligned their long axes parallel to the groove direction. This alignment was dependent on the groove depth, with increasing groove depth increasing guided cell alignment. When cells were cultured in a physiological dc electric field (EF) on non-grooved, flat surfaces, the cells aligned in response to the EF, with their long axes perpendicular to the EF vector. This response was EF strength dependent, increasing EF strength (from 20 to 200mV/mm) increased cell alignment, perpendicular to the EF vector. These two guidance cues were applied simultaneously, so that the EF vector was parallel to the groove direction. At high but still physiological EF strengths (200mV/mm) cells ignored the topography and were guided by the EF alone, aligning perpendicular to the EF vector, as on non-grooved surfaces. At low field strengths (20mV/mm) cells responded only to the topographic guidance cue, with cells aligning parallel to the grooves and therefore also to the EF vector. Intermediate field strengths (50 to 100mV/mm) produced a mixed response, with cells responding to both guidance cues. The effect of removing serum from the culture medium on the EF and topographical guidance of fibroblast cells was studied and the results were compared to cells on non-grooved surfaces. Removal of serum produced a small but significant decrease in the angle of cell alignment for cells on non-grooved surfaces, from 78 to 63 degrees, relative to the EF vector, but did not completely suppress the EF guidance cue. In contrast, the EF guidance of cells on grooved substrates was suppressed almost completely by the absence of serum, with cells responding only to the grooved topography, aligning their long axis parallel to the grooves and the EF vector. These results imply that alignment of fibroblasts by topography is serum-independent, but alignment by EFs is serum-dependent. In addition they demonstrate that the alignment of fibroblast cells can be tailored by the dual guidance cues of topography and electric fields.

INTRODUCTION

The morphology of fibroblasts can be directed by various guidance cues. The control of cell alignment is important in the design of tissue-engineered implants to replace tendons and ligaments, which naturally exhibit alignment of cells in parallel to collagen fibres. Of the various guidance cues, contact guidance, whereby cells respond to nano- and micro-scale topography, has been studied most extensively [1,2], and is possibly the most relevant to tissue engineering. In general, cells become highly oriented along grooves with widths between 1 and 10 μm. There is some disagreement, however, as to whether groove depth is the most significant factor in guiding cell alignment [3,4]. Another physiologically relevant guidance cue is an electric field (EF), which is present in many tissues during development and wound repair [5]. Fibroblasts

respond to a d.c. EF by aligning their long axes perpendicular to the direction of the EF vector [6-8], and migrating towards the cathode [6,7], although a recent study showed that EFs do not direct the migration of fibroblast cells cultured on collagen I [9].

As these two guidance cues may occur simultaneously *in vivo*, e.g. during skin wound healing, and may also be used in guided tissue growth *in vitro*, we aimed to study the effect of presenting fibroblast cells with these conflicting guidance cues.

EXPERIMENTAL DETAILS

Ligaments were dissected in sterile conditions from the bovine carpo-metacarpal joints of animals less than two years old, obtained from a local abattoir. The ligament was washed in sterile PBS, cut into small pieces, and placed in 25cm^2 tissue culture flasks with 12ml of culture medium (DMEM (31885-023), supplemented with 10% foetal bovine serum and penicillin/streptomycin – all from Invitrogen Ltd., UK) at 37°C in 5% CO_2; during culture, the medium was replaced every 2-3 days. After 72-96 hours, a significant number of fibroblast cells had migrated out of the ligament pieces, so the ligament was removed. When cells approached confluence they were sub-cultured by firstly washing with PBS, and then adding trypsin-EDTA; subsequent cultures were carried out in 80cm^2 flasks. Cells were used between passage 4 and 10.

The preparation of the micro-grooved substrates used in this study has been described extensively elsewhere [10]. Briefly, a series of repeating, parallel grooves and ridges were etched on the surface of fused quartz microscope slides by a direct writing electron beam lithographic process. Slides with a total of 12 different groove depth and width combinations were produced (groove/ridge widths of 1, 2 and 4 μm, and groove depths of 62, 347, 547 and 1024nm), with each condition comprising of a 5 x 5 mm block of grooves. Before use for cell culture, the slides were soaked overnight in concentrated HNO_3, and then washed repeatedly with deionised water. The slides were then sterilised using UV irradiation. Fibroblasts ($5x10^4$) were seeded on to the etched micro-grooved surfaces in 2ml of culture medium and allowed to attach overnight.

A detailed description of the experimental set-up for applying d.c. electric fields across the cell culture has been described previously [10]. Briefly, two 50 x 10 mm glass coverslips were fixed in parallel, with a spacing of 10 mm between them, on to a plastic Petri dish with DC4 silicone sealant (Dow Corning). Excess medium was removed from the micro-grooved substrate, and the substrate was fixed face down on to the parallel coverslips with DC4 sealant. This resulted in the cells on the grooved substrate being exposed to a 'tunnel' of medium that was 50 x 10 x 0.5 mm in dimensions, with the grooves running in parallel to the direction of the applied electric field. Two reservoirs were formed at either end of the coverslip/substrate channel with DC4 sealant, and these were filled with medium. A d.c. EF was delivered from a power supply, with the two electrodes connected to Ag/AgCl wires that were dipped into beakers of Steinberg's solutions. These beakers were then connected to the two reservoirs of medium in the culture dish by agar-salt bridges; this set-up avoids contamination of the culture medium from electrode products. Field strengths were measured with a digital voltmeter at the beginning and end of the experiments. The whole set-up was placed in an incubator at 37°C in 5% CO_2. EFs were applied for 24 hours, with images taken before and after this period using a Leica Image Analyser (Q500MC, Leica). Data were pooled from at least three repeat experiments for each condition.

The alignment of cells was determined by drawing a line through the cell long axis, and measuring the angle between this line and the x-axis, which was parallel to the groove/EF

direction, with values ranging from 0 to 90°. Therefore, angles of 0° and 90° correspond to cells aligned in parallel and perpendicular to the grooves/EF, respectively, and a population of cells with an average angle of 45° will be randomly oriented. Statistical analysis was performed using unpaired student's t-tests. Data are expressed as mean ± s.e.m., unless otherwise stated.

RESULTS and DISCUSSION

When fibroblast cells were exposed to the micro-grooved surfaces without an applied electric field, they oriented their long axes parallel to the direction of the grooves, as in other studies [e.g. 1-4]; the response did depend on groove dimensions, Figure 1. Deeper grooves directed cell alignment more strongly than shallow grooves, with cells growing on 62 nm deep grooves exhibiting close to a random (45°) response. For the deeper grooves (547 and 1024 nm) cell alignment did not depend on the groove width, whereas for each of the shallower grooved substrates (62 and 347 nm) a significant difference in cell alignment for the three different groove widths was observed.

Cells grown on flat, non-grooved surfaces were exposed to an EF of 200mV/mm. From the cell population (n=339), 84% of cells aligned perpendicular, at angles of >80°, to the EF vector, in agreement with other studies [6-8]. Figure 2 summarises these difference in cell alignment between cells exposed to grooves only (depth of 347 nm and width of 2 μm) and EFs only. Each of the two guidance cues resulted in polarised cell alignment but, as the cues were oriented in the same direction, they resulted in orthogonal responses.

The response of cells to the two guidance cues simultaneously is shown in Figure 3; the EF strength was 200mV/mm and the groove depth and width were 547 nm and 4 μm, respectively. For these conditions, the cells responded predominantly to the EF as a guidance cue, with their long axes aligned perpendicular to the EF/groove direction.

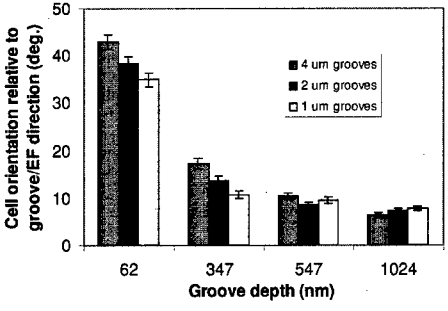

Figure 1. Cell alignment on grooved substrates in the absence of an EF; the extent of cell alignment depended on the groove depth, with deep grooves guiding cells more strongly (for each condition, n=247).

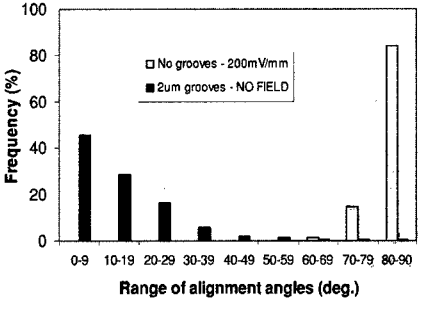

Figure 2. The distribution of cell alignment responding to either grooves or EF; EFs align cells perpendicular to the groove/EF direction.

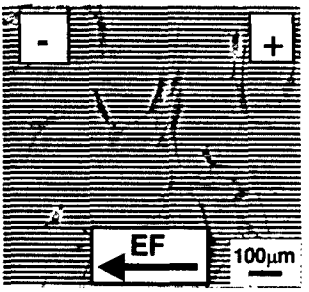

Figure 3. The response of cells exposed simultaneously to grooves and an EF (200mV/mm); cells align their long axes perpendicular to the groove/EF direction.

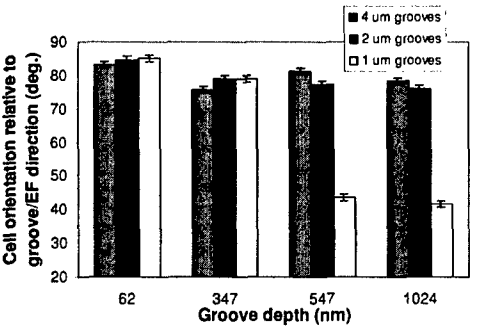

Figure 4. The effects of groove depth and width on the alignment of cells exposed to an EF (200mV/mm). For most conditions, cells responded only to the EF as a guidance cue and ignored the grooves (for each condition, n=153).

The effect of the different groove dimensions on the alignment of cells presented with both guidance cues simultaneously is illustrated in Figure 4. For most groove dimensions, cells were directed by the EF and ignored the guidance cue offered by the grooves; cells aligned their long axes perpendicular to the EF/groove direction. The grooves only guided cells on two groove dimensions, depths of 547 and 1024 nm with groove widths of 1 μm, but the cell guidance was clearly a mixture of the two cues. The distribution of alignment angles for these two conditions was neither uni-modal nor bi-modal, but rather an even distribution of angles between 0 and 90°, comparable to the control conditions of no grooves and no EF (data not shown).

The test the hypothesis that the EF strength of 200mV/mm was too dominant a guidance cue, the effect of smaller EF strengths on the alignment of cells on grooved substrates was studied. A cell depth of 347 nm was chosen for this study as this dimension demonstrated contact guidance of cells in the absence of an EF (Figure 2) but showed consistent EF-guidance of cells at 200mV/mm for all groove depths (Figure 4). The data for cells grown on these substrates at field strengths of 20, 50, 100 and 200 mV/mm are shown in Figure 5a; cells grown on flat, non-grooved surfaces at the same EF strengths are included as a control. Firstly, the alignment of cells grown on the non-grooved surfaces showed a clear dependence on EF strength, with cells exposed to an EF of 20 mV/mm exhibiting only a weak perpendicular response that was close to random (51.0° relative to the groove/EF direction, statistically different from the random response for no EF). Increasing the EF strength up to 200mV/mm resulted in a steady increase in the alignment angle of cells. For cells grown on the grooved surfaces, the results could be summarised as three different responses. For low EF strengths (20mV/mm), cells did not appear to respond to the EF, but were guided by the grooves, aligning in a direction that approached being parallel to the groove/EF direction. The alignment of these cells at 20 mV/mm was similar to the no field results shown in Figure 1. For high EF strengths (200mV/mm), cells were guided exclusively by the EF, ignoring the grooved surfaces, as discussed previously for Figure 4. Intermediate field strengths (50 and 100 mV/mm) resulted in a mixed response, with angles between 30 and 55°.

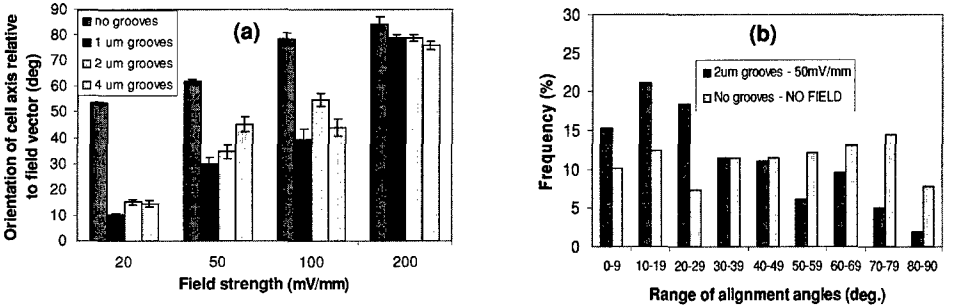

Figure 5. (a) The effect of EF strength on the alignment of cells grown on surfaces with a groove depth of 347 nm and groove widths of 1, 2 and 4 μm. Cells grown on flat, non-grooved surfaces are included for comparison. (b) the distribution of alignment angles of cells (n=261) grown on a grooved surface (347 nm depth and 2 μm width) with an EF strength of 50mV/mm; cells grown on non-grooved surfaces with no EF are included for reference.

To determine if this mixed response was due to the averaging of the results of two distinct populations of cells responding individually to the two guidance cues, or if these intermediate field strengths were producing a random distribution of cell alignments, the cell alignments were grouped into nine 10° groupings between 0 and 90° and their frequencies plotted, Figure 5b. The distribution of alignment angles for cells grown on flat, non-grooved surfaces with no EF are included to illustrate a typical random distribution. These results for an EF strength of 50mV/mm and a groove depth and width of 347 nm and 2 μm, respectively, showed that the distribution was not random, with a large population of cells responding to the grooves (between 0 and 29°) and a smaller population responding to the EF (between 60-69°). It should be noted, however, that these two populations did not constitute narrow, isolated groupings, as many cells (~30%) aligned at angles between 30 and 59°, which is out-with the range expected for each of the guidance cues acting alone, see Figure 2.

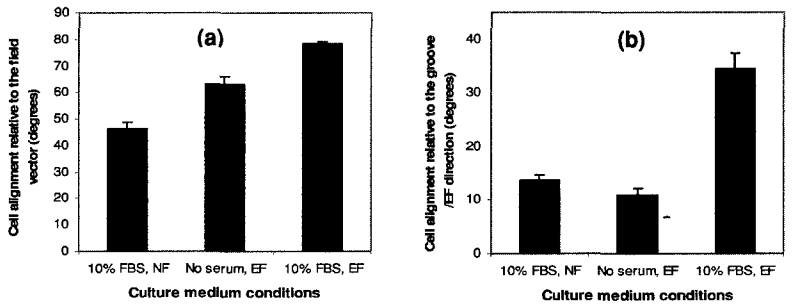

Figure 6. The effect of removing serum (FBS) from the culture medium on cell alignment on (a) non-grooved and (b) grooved (347 nm depth, 2 μm width) surfaces, with an EF of 100mV/mm. A control of cell alignment with serum but with no EF (NF) is included for comparison.

The results described in Figures 3-5 have shown that when the EF strength is sufficiently large, it is the dominating guidance cue. To begin to understand the mechanisms involved in this EF-dominated response, a number of experiments were repeated using serum-free medium. Here, cells were seeded onto the substrates (both non-grooved and with grooves of 347 nm depth and 2 μm width) with serum-free medium, allowed to attach overnight, and either complete medium (with 10% FBS) or serum-free medium added for the EF experiment (100mV/mm). Figure 6a shows that for cells grown on flat, non-grooved surfaces with serum removed, the angle of cell alignment was significantly reduced, although not completely suppressed, compared to the control with serum but no EF. For cells grown on the grooved surfaces, Figure 6b, the EF-guided response that was observed with serum was completely suppressed when serum was removed, with cells being guided by the grooved surface only; the response of cells grown on the same groove dimensions with serum added but without an EF is included for comparison. These results imply that the EF-dominated guidance of cells on grooved surfaces is serum dependent, and this may be related to the presence of one or more growth factors.

CONCLUSIONS

When grooved surfaces and electric fields are presented simultaneously as guidance cues to bovine ligament fibroblasts, the cells align their long axes in response to the EF for large field strengths (200mV/mm), but ignore the EF and align in response to the grooves for small fields (20mV/mm). The EF-dominated response was shown to be serum dependent, which suggests a mechanism that requires growth factor receptor activation.

ACKNOWLEDGEMENTS

The authors thank Dr Ann Rajnicek and Professors Curtis and Wilkinson for use of the micro-grooved substrates, and the Department of Orthopaedics, University of Aberdeen for obtaining bovine ligaments for producing fibroblast cultures. The research was supported by a Scottish Higher Education Funding Council (SHEFC) Research Development Grant (RDG).

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